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Interferon-γ Activates Expression of p15 and p16 Regardless of 9p21.3 Coronary Artery Disease Risk Genotype

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Objectives Because post-transcriptional mechanisms modulate levels of p16 (encoded by *CDKN2A*) and p15 (encoded by *CDKN2B*), we tested whether interferon-γ regulates the expression of these proteins and the effect of the 9p21 genotype.

Background The mechanism whereby the common variant at chromosome 9p21.3 confers risk for coronary artery disease (CAD) remains uncertain. A recent report proposed that 9p21.3 confers differential activation of adjacent genes in response to interferon-γ, and reported that mRNA levels of *CDKN2B* are reduced in response to interferon-γ.

Methods Human umbilical vein endothelial cells (HUVECs), aortic smooth muscle cells, HeLa cells, HEK293 cells, and 16 human lymphoblastoid cell lines, all genotyped for the 9p21.3 locus, were treated with interferon-γ and analyzed by immunoblot.

Results In all cells tested—except HUVECs where expression was not modulated by interferon-γ—regardless of 9p21.3 genotype, interferon-γ increased the expression of p16 and p15. Northern blot analysis confirmed that interferon-γ has little effect on mRNA levels of *CDKN2A* and *CDKN2B*.

Conclusions The 9p21.3 risk genotype does not affect the activation of cyclin-dependent kinase inhibitors p15 and p16 by interferon-γ. Thus, another mechanism is likely to account for the CAD risk associated with this locus. (J Am Coll Cardiol 2013;61:143–7) © 2013 by the American College of Cardiology Foundation

The common genetic variant located in the vicinity of the genes encoding the cyclin-dependent kinase inhibitors p15 (*CDKN2B*) and p16/ARF (*CDKN2A*) on the short arm of chromosome 9 at 9p21.3 contributes to the risk of CAD by an unknown mechanism that is independent of known risk factors (1–3). 9p21.3 associates with the severity of coronary atherosclerosis in a risk allele–dosage-specific manner (4), recently confirmed by others (5). Among cases with coronary artery disease (CAD), 9p21.3 does not associate with myocardial infarction (4), suggesting that it promotes atherosclerosis rather than thrombosis.

The 9p21.3 risk locus consists of many tightly linked single nucleotide polymorphisms (SNPs) that cover an area of about 58,000 bp. Whether a single SNP at this locus is functional, or whether several SNPs have a functional effect, remains controversial. We identified enhancer sequences within the 9p21.3 region, and in particular, we found that the SNP rs1333045 conferred differential enhancer activity (6). A recent study that ablated the homologous sequences in the mouse genome observed markedly reduced expression of *CDKN2A* and *CDKN2B* mRNA (7), confirming the existence of regulatory sequences in this region. Another recent report provided evidence that the SNP rs10757278 at the 9p21.3 CAD risk locus disrupts a STAT1-responsive sequence and confers differential activation of adjacent genes in response to interferon-γ (8). Thus, these authors suggested that inability to up-regulate p15 and p16 would promote cellular proliferation and atherosclerosis. However, the evidence they provided to support this suggestion appeared to contradict their hypothesis. For example, quantitative polymerase chain reaction (PCR) measured reduced

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Abbreviations and Acronyms

CAD = coronary artery disease

HAoSMC = human primary aortic smooth muscle cells

HUVEC = human umbilical vein endothelial cell

LCL = lymphoblastoid cell

PCR = polymerase chain reaction

SNP = single nucleotide polymorphism

mRNA for *CDKN2B* in response to interferon-γ in HeLa cells and human umbilical vein endothelial cells (HUVECs), both heterozygous for the rs10757278 SNP (8). However, the single STAT-1–responsive allele in heterozygous cells should have been activated by interferon-γ. Moreover, altered mRNA expression does not always translate to altered protein levels. For example, transforming growth factor-β markedly elevates p15 protein levels but barely increases mRNA levels (9), whereas the micro-RNA miR-24 suppresses translation of the p16 mRNA without affecting mRNA levels (10). In light of these post-transcriptional mechanisms, and to confirm whether or not an interferon-γ–dependent mechanism operates at the 9p21.3 risk locus, we re-examined the association of the 9p21.3 genotypes with the response of p15 and p16 to interferon-γ at the protein level in different cell lines.

Materials and Methods

Cell cultures. Human primary aortic smooth muscle cells (HAoSMCs) were purchased with culture medium from Cell Applications (San Diego, California); HUVECs were obtained from Lonza (Allendale, New Jersey), and cultured as recommended; and lymphoblastoid cells (LCLs) (n = 16) were selected for their 9p21 genotype and purchased from the Coriell Institute for Medical Research (Camden, New Jersey), and cultured in RPMI Medium 1640 (Invitrogen, Burlington, Ontario, Canada). Recombinant human interferon-γ was purchased from R&D Systems (Minneapolis, Minnesota).

Genotyping. DNA from HeLa, HUVECs, HEK293 cells, and LCLs was genotyped for the 9p21.3 risk locus by PCR and sequencing. A DNA fragment containing SNP rs1333048 was obtained using primers 5′-TGG CTA TAA ATG CCT TTG GC-3′ and 5′-CCA TAT ATC TTG CTT ACC TCT GCG-3′, and another fragment containing rs10757278 and rs10811656 was obtained using primers 5′-TTG GAA CTG AAC TGA GGC CAG ACA-3′ and 5′-TAG ACT CCA CGC TGT TCC CAA GTA-3′. HAoSMCs were genotyped on Affymetrix Axiom arrays (Affymetrix, Santa Clara, California).

Processing of cells for immunoblotting. Cells were lysed in RIPA buffer (4.25 mmol/l Tris [pH 8.0], 135 mmol/l NaCl, 1% IGEPAL CA-630, 1% SDS 0.5% deoxycholate) containing protease and phosphatase inhibitors (Roche Molecular Systems, Branchburg, New Jersey). Lysate protein concentrations were measured by Bradford assay before SDS-PAGE and immunoblot analysis.

Antibodies and immunoblot analyses. Mouse anti-p16 (sc-9968) and anti-p15 (sc-171798) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California), antibody to p14 (clone 4C6/4) was from EMD Millipore (Temecula, California), antibody to phosphorylated serine 727 (p-Ser 727) of Stat1 from Cell Signaling Technology (Danvers, Massachusetts), goat anti-mouse IgG (HAF007) from R&D Systems, and goat anti-rabbit IgG (#31460) from Thermo Fisher Scientific (Rockford, Illinois). Immunoblots were quantified and normalized to GAPDH levels using ImageQuant TL v2005 software version 7.0 (GE Healthcare, Little Chalfont, United Kingdom), expressed as mean ± SD.

Northern blot analysis. RNA was isolated from HeLa and HUVECs treated with vehicle (phosphate-buffered saline with 0.1% bovine serum albumin) or with 100 ng/ml interferon-γ using TRIzol reagent (Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. Fifteen micrograms of RNA was size fractionated by polyacrylamide gel electrophoresis, transferred to a nylon membrane, and then probed with *CDKN2A* (clone 3954155, Open Biosystems, Lafayette, Colorado) or *CDKN2B* (clone 4871014) radiolabelled with $dCT^{32}[P]$ using the random primers technique (Agilent Technologies, La Jolla, California). Images were revealed using a phosphor-storage screen and a Storm 860 molecular imager (GE Healthcare Life Sciences, Baie D'Urfe, Quebec, Canada).

Results

Interferon-γ markedly increases p16 and p15 protein levels. LCLs are Epstein-Barr virus transformed B lymphocytes that remain in a proliferative state because $p16^{INK4A}$ (*CDKN2A*) expression is repressed by the Epstein-Barr virus nuclear antigens 3A and 3C (11,12). The ability to immortalize B lymphocytes from many individuals of different ancestry and to ascertain by high throughput the genotypes of common genetic variants has been a cornerstone of the International HapMap project (13). Because the genotype of common variants, including the rs10757278, are known or can be imputed for these cells lines, and because these cells maintain a low level of p16 expression, we tested whether interferon-γ can activate the expression of p16 and p15 and whether the 9p21.3 CAD risk genotype influences this response.

LCLs for each 9p21.3 genotype were selected from the HapMap database and purchased from the Coriell Institute for Medical Research. Where the genotype for the STAT1-responsive sequence disrupted by rs10757278 was not known, it was imputed using linked flanking SNPs ($D' = 1.0$, $r^2 > 0.8$) (Table 1). All cell lines were genotyped for the rs10757278 SNP by PCR amplification and direct sequencing, and the genotypes agreed with the imputation. LCLs treated with interferon-γ markedly increased the levels of p15 and p16, as revealed by immunoblot (Fig. 1A). We were unable to detect the alternative product of the *CDKN2A* gene, $p14^{ARF}$ (data not shown). Importantly, of

Table 1 Imputation and Sequence Confirmation of rs10757278 Genotypes of the Lymphoblastoid Cells Used in This Study

LCL ID	rs944797	rs2383207	rs1537375	rs10757278	rs1333048	rs1333049
GM10835	CC	GG	CC	GG	CC	CC
GM11992	CC	GG	CC	GG	CC	CC
GM11995	CC	GG	CC	GG	CC	CC
GM12282	CC	GG	CC	GG*	CC	CC
GM11840	CT	AG	CT	AG	AC	CG
GM12234	CT	AG	CT	AG	AC	CG
GM12817	CT	AG	CT	AG*	AC	CG
GM12864	CT	AG	CT	AG	AC	CG
GM12872	CT	AG	CT	AG	AC	CG
GM12873	CT	AG	CT	AG	AC	CG
GM12003	TT	AA	TT	AA*	AA	GG
GM12057	TT	AA	TT	AA	AA	GG
GM12283	TT	AA	TT	AA*	AA	GG
GM12336	TT	AA	TT	AA*	AA	GG
GM12775	TT	AA	TT	AA*	AA	GG
GM12890	TT	AA	TT	AA*	AA	GG

*Imputed genotypes using flanking single nucleotide polymorphisms, sorted by relative position on chromosome 9, with a D' = 1 and an $r^2 > 0.8$ were confirmed by sequencing.
LCL = lymphoblastoid cells.

the 16 LCLs tested (5 homozygous nonrisk, 5 heterozygotes, and 6 homozygote risk for the 9p21.3 genotype), regardless of 9p21.3 CAD genotype, no substantial difference was observed in the level to which interferon-γ increased p15 (n = 16) and p16 (n = 12) expression (Fig. 1B).

The response to interferon-γ was also tested in other cell types (Fig. 2A). In HAoSMCs heterozygous for the rs10757278 genotype (as well as for rs1333045, rs1333048, and rs133049), a robust elevation of p15 and p16 was observed. By contrast, p15 and 16 levels were not affected by interferon-γ treatment in HUVECs homozygous for the nonrisk genotype of 9p21.3 (rs1333048), despite a clear activation of pSTAT1. Similar to the LCLs, the cell lines HEK293 (homozygous for the 9p21.3 rs1333048 risk allele) and HeLa (heterozygous for rs1333048) also increased p16 and p15 levels in response to interferon-γ. To determine whether interferon-γ treatment affects *CDKN2A* and *CDKN2B* mRNA levels, we performed Northern blot analysis of HUVECs and HeLa cells (Fig. 2B). Interferon-γ did not elevate CDKN2A levels in HeLa cells, and *CDKN2A* mRNA was not detected in HUVECs. On the other hand, a slight increase in *CDKN2B* was detected in HeLa cells treated with interferon-γ, whereas there was no change in *CDKN2B* mRNA in HUVECs.

Discussion

A recent study claimed that the mechanism whereby the 9p21.3 risk locus operates is by impairing the response to interferon-γ (8). These authors reported that interferon-γ markedly reduced the mRNA levels of the cyclin dependent kinase inhibitor p15 (encoded by *CDKN2B*) in HeLa and HUVECs (cells that are heterozygous for the 9p21.3 risk locus) (8). However, using the same dose of interferon-γ (100 ng/ml), we observed increased expression of p15 in all cell types examined except HUVECs, where the levels were unchanged. Importantly, when tested in 16 different LCLs, the 9p21.3 risk locus did not affect the response of p15 and p16 to interferon-γ. Thus, it seems unlikely that the rs10757278 SNP that disrupts a STAT1-responsive element in the 9p21.3 risk locus is sufficient to affect the activation of p15 and p16 in response to interferon-γ. It is worthwhile pointing out that whereas Harismendy et al. (8) measured *CDKN2B* mRNA by reverse-transcription PCR, we measured protein levels of p15 and p16 by immunoblot. Furthermore, Northern blot analysis of mRNA from HeLa cells (heterozygote for 9p21.3) showed no change in *CDKN2A* mRNA and a slight increase in *CDKN2B* mRNA. In addition, our HUVECs were homozygous for the nonrisk allele of 9p21.3, thus should have responded robustly to interferon-γ, but showed no change in *CDKN2B* mRNA levels. Both results contrast with the reduced expression reported by Harismendy et al. Our study is consistent with a previous report that also found that p16, but not $p14^{ARF}$, is up-regulated by interferon-γ in human cells (14). Furthermore, interferon-γ was reported to increase the translation of the ICAM1 protein by a STAT1-dependent mechanism that suppresses miR-221 expression (15). Thus, in some instances, interferon-γ can increase protein translation, and in the case of p16, this effect appears to be independent of the mRNA levels. Our study indicates that the mechanism whereby the 9p21.3 risk allele contributes to CAD is not by blocking the activation of p15 or p16 by interferon-γ.

Multiple enhancer-like sequences are present within the 9p21.3 risk locus that could influence the expression of flanking genes (6–8). Under basal conditions, in nontransformed cells, there may be differences in the levels of p15

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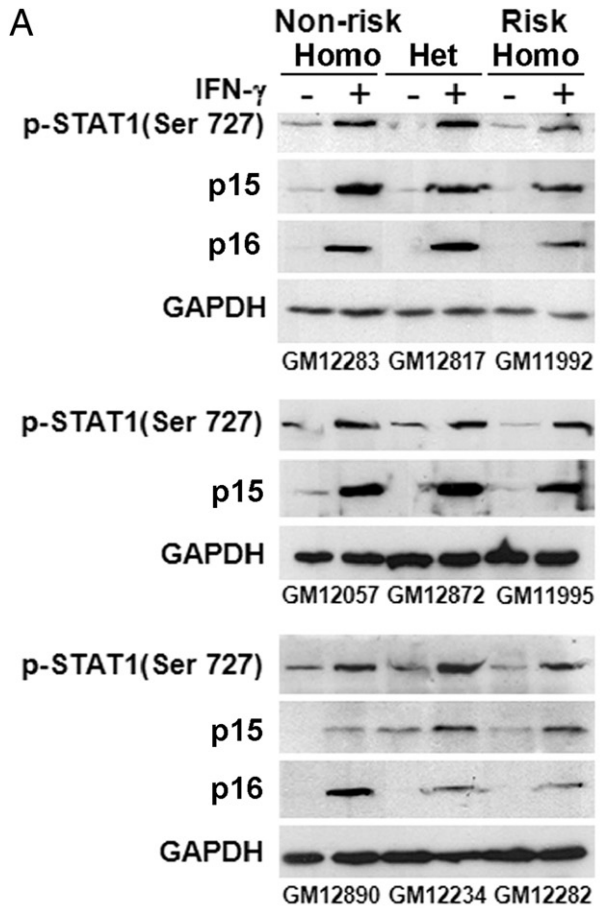

Figure 1 **Interferon-γ Activates p16 and p15 Expression Regardless of 9p21.3 Genotype**

(A) Lymphoid cells (LCLs) homozygous (Homo) nonrisk (n = 5), heterozygote (Het) (n = 5), or homozygote risk (n = 6) for the 9p21.3 genotypes were treated with vehicle or 100 ng/ml recombinant human interferon-γ (INF-γ) for 24 h and then harvested for immunoblot analysis (representative results). Antibody to phosphorylated serine 727 (p-Ser 727) of Stat1 confirmed activation by interferon-γ. Numbers below lanes are unique identifiers for the LCLs. **(B)** Quantification of the response to interferon-γ showed no substantial difference between genotypes for p15 (n = 5 per genotype) or p16 (n = 3 per genotype). Fold induction is expressed as mean ± SD.

and/or p16 conferred by the enhancer sequences at the 9p21.3 locus. However, these basal differences may be masked by the repression of the *CDKN2A* locus by the Epstein-Barr virus nuclear antigens 3A and 3C (11,12). Our choice of LCLs was not to test basal differences in p15 and p16 expression but rather to test whether the 9p21.3 genotype influences the response of these genes to interferon-γ.

The hypothesis that the 9p21.3 locus confers a differential response to interferon-γ is clearly not supported by our data, because we observed increased p15 and p16 expression regardless of genotype. The disease process itself may influence the activity of the enhancer sequences at 9p21.3. Many other growth factors and cytokines are produced at atherosclerotic lesions (16) and could have differential effects at other enhancer elements that contain functional SNPs, like the one we identified at rs1333045 (6). In addition, many SNPs at the 9p21.3 locus could be the functional culprits and may act together to modulate gene expression. For example, the linked polymorphism rs10811656 located only 4 bp from the putative STAT1 binding sequence (rs10757278) disrupts a putative TEAD factor binding site (CATTCCG>CATTCTG) (17), but it is not known whether this element is functional or influences enhancer activity. TEAD factors are known to regulate cell cycle progression (18,19), and it will be interesting to see whether

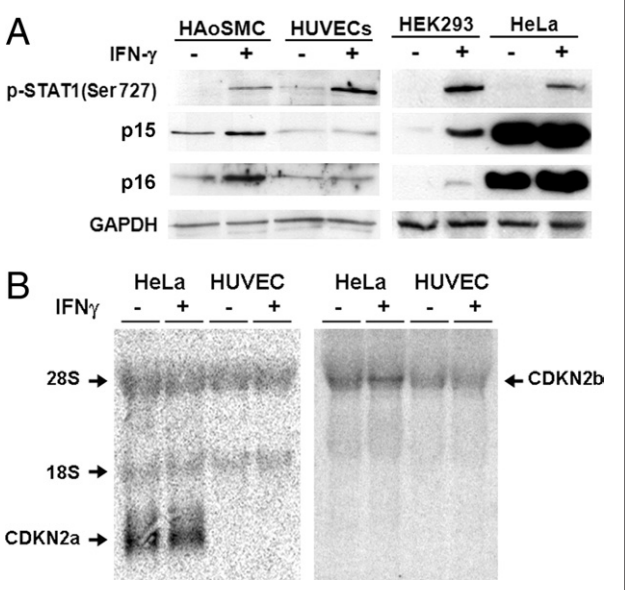

Figure 2 **Effect of Interferon-γ on p15 and p16 Levels in Different Cell Types**

(A) Results of immunoblot analysis of protein extracts from human primary aortic smooth muscle cells (HAoSMCs) (9p21 heterozygote) and human umbilical vein endothelial cells (HUVECs) (9p21 homozygote nonrisk), as well as from HEK293 (homozygote risk) and HeLa (heterozygote) cells are shown. GAPDH was not affected by interferon-γ and was used as a loading control. **(B)** Northern blot analysis shows no or only modest increases in *CDKN2A* and *CDKN2B* levels in HUVECs and HeLa cells in response to interferon-γ (INF-γ). 28S and 18S RNA signals confirmed equal loading of 15 μg of RNA per lane.

differential binding of TEAD factors might account for the risk of coronary artery disease in future studies. Of the more than 100 SNPs linked to the 9p21.3 locus, exhaustive functional characterization would be required and is beyond the scope of the present study. Thus, the precise mechanism whereby the 9p21.3 locus contributes to the risk of CAD remains to be elucidated.

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REFERENCES

1. McPherson R, Pertsemlidis A, Kavaslar N, et al. A common allele on chromosome 9 associated with coronary heart disease. Science 2007; 316:1488–91.
2. Helgadottir A, Thorleifsson G, Manolescu A, et al. A common variant on chromosome 9p21 affects the risk of myocardial infarction. Science 2007;316:1491–3.
3. Samani NJ, Erdmann J, Hall AS, et al. Genomewide association analysis of coronary artery disease. N Engl J Med 2007;357:443–53.
4. Dandona S, Stewart AFR, Chen L, et al. Gene dosage of the common variant 9p21 predicts severity of coronary artery disease. J Am Coll Cardiol 2010;56:479–86.
5. Chan K, Motterle A, Laxton RC, Ye S. Common variant on chromosome 9p21 predicts severity of coronary artery disease. J Am Coll Cardiol 2011;57:1497–8; author reply 1498–9.
6. Jarinova O, Stewart AFR, Roberts R, et al. Functional analysis of the chromosome 9p21.3 coronary artery disease risk locus. Arterioscler Thromb Vasc Biol 2009;29:1671–7.
7. Visel A, Zhu Y, May D, et al. Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice. Nature 2010;464:409–12.
8. Harismendy O, Notani D, Song X, et al. 9p21 DNA variants associated with coronary artery disease impair interferon-gamma signalling response. Nature 2011;470:264–8.
9. Sandhu C, Garbe J, Bhattacharya N, et al. Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells. Mol Cell Biol 1997;17:2458–67.
10. Lal A, Kim HH, Abdelmohsen K, et al. p16(INK4a) translation suppressed by miR-24. PLoS One 2008;3:e1864.
11. Maruo S, Zhao B, Johannsen E, Kieff E, Zou J, Takada K. Epstein-Barr virus nuclear antigens 3C and 3A maintain lymphoblastoid cell growth by repressing p16INK4A and p14ARF expression. Proc Natl Acad Sci U S A 2011;108:1919–24.
12. Skalska L, White RE, Franz M, Ruhmann M, Allday MJ. Epigenetic repression of p16(INK4A) by latent Epstein-Barr virus requires the interaction of EBNA3A and EBNA3C with CtBP. PLoS Pathog 2010;6:e1000951.
13. International HapMap Consortium. The International HapMap Project. Nature 2003;426:789–96.
14. Chaturvedi V, Cesnjaj M, Bacon P, et al. Role of INK4a/Arf locus-encoded senescent checkpoints activated in normal and psoriatic keratinocytes. Am J Pathol 2003;162:161–70.
15. Hu G, Gong AY, Liu J, Zhou R, Deng C, Chen XM. miR-221 suppresses ICAM-1 translation and regulates interferon-gamma-induced ICAM-1 expression in human cholangiocytes. Am J Physiol Gastrointest Liver Physiol 2010;298:G542–50.
16. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 2006;86:515–81.
17. Stewart AFR, Larkin SB, Farrance IKG, Mar JH, Hall DE, Ordahl CP. Muscle-enriched TEF-1 isoforms bind M-CAT elements from muscle-specific promoters and differentially activate transcription. J Biol Chem 1994;269:3147–50.
18. Benhaddou A, Keime C, Ye T, et al. Transcription factor TEAD4 regulates expression of myogenin and the unfolded protein response genes during C2C12 cell differentiation. Cell Death Differ 2012;19: 220–31.
19. Cao X, Pfaff SL, Gage FH. YAP regulates neural progenitor cell number via the TEA domain transcription factor. Genes Dev 2008; 22:3320–34.

Key Words: 9p21.3 ■ coronary artery disease ■ cyclin-dependent kinase inhibitor ■ interferon-γ ■ p15 ■ p16.